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Improving the esterification activity of *Pseudomonas cepacea* and *Burkholderia cepacia* lipases *via* cross-linked cyclodextrin immobilization

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Abstract

The search for a new, efficient and sustainable matrix for biocatalyst immobilization is a growing area in biotechnology. Our proposed approach deals with the utilization of solid cross-linked b-cyclodextrin as supports for enzyme immobilization. Results obtained in terms of enzyme activity and thermal stability of novel immobilised materials have been found to remarkably improve those obtained using commercial immobilized enzymes in esterification reactions (e.g., monostearin synthesis).

Introduction

Lipases are triacylglycerol acyl hydrolytic enzymes that have found use as hydrolysis, esterification and transesterification reaction catalysis.[1] Upon immobilization, supported enzymes can provide an easily separable and reusable system (together with enhanced product recovery) which boasts of enhanced resistance to deactivation as compared to free enzymes.[2] Immobilization has several implications when generating increasingly stable biocatalysts compatible with continuous processing technologies.[3] Various strategies to immobilize enzymes on a number of supports have been reported. These range from the more extended and widely employed physical methods (e.g. adsorption, entrapping and/or electrostatic immobilization) to chemical protocols (e.g. covalent immobilization).[4]

Cyclodextrins (CDs) are a class of macrocyclic structures comprising (α-1,4)-linked β-D-glucopyranose units that possess a relatively non-polar cavity. The internal hydrophobic cavity and the external hydrophilic rim of CDs render them ideal for modelling host–guest interactions,[5] drug delivery,[6] catalysis,[7, 8] chiral separation,[9] and molecular recognition in self-assembled monolayers.[10] β-CD has proven to be a good enzyme support, with a number of contributions reporting significant efficiency in promoting catalytic processes both in water and organic solvents. The addition of β-CD to solutions containing lipases has been reported to enhance reaction rates as well as enantioselectivity and lipase stability.[11] Furthermore, CD immobilized *Candida rugosa* lipase offered important advantages (e.g. thermal stability) over its free enzyme counterparts.[12]

Biodiesel which includes alkyl esters of long chain fatty acids has been proposed as a suitable bio-derived replacement for petroleum diesel as a means to reduce gaseous pollutant emissions, such as CO, SO_x, and organic compounds.[13] The properties of biodiesel are similar to those of petroleum-based diesel, allowing its use either as a substitute for diesel fuel or more commonly in fuel blends. Several strategies for biodiesel production have been reported in recent years and include homogeneous/ heterogeneous[14] and biocatalytic triacylglyceride transesterification protocols with most commonly extended low molecular weight alcohols .[15]

Mono- and diacylglycerols (MAG and DAG) are well-known biodegradable, biocompatible, nontoxic and nonionic surfactants widely used in food, pharmaceutical and industrial applications.[16] The hydrophobic part consists of fatty acids (i.e., lauric, myristic, palmitic, oleic and stearic acid), whereas the hydrophilic part can be formed of glycerol or one of its ester derivatives of organic acids such as lactic, citric, acetic or tartaric acid. They are commonly produced on the basis of the batch alkaline catalyzed chemical glycerolysis of natural oil and fats at high temperatures (220°-250°C) and elevated pressure under nitrogen atmosphere. Besides the high energy consumption of their preparation, high temperatures are responsible for low yields (<50%) and poor product quality which leads to dark-coloured and burned-tasting product formation, thus requiring costly and extensive purification steps.

Comparably, biocatalysis can overcome these issues and lead to an environmentally friendly approach for MAG synthesis via selective hydrolysis or alcoholysis using 1,3-regiospecific lipases,[17] esterification of glycerol with fatty acids,[18] and glycerolysis of fats or oils.[13,15] Monostearin stands out as one of most relevant available

monoacylglycerols due to its numerous applications as an additive in foodstuffs (e.g. candies, ice cream, cakes and bread), and its important role as an emulsifier, disperser, anti-frothing agent and preservative enhancer.

Following recent research of the groups into green chemical protocols and innovative biotechnological strategies,[19] we herein report an easy and reproducible one-pot sonochemical β-CD reticulation in the presence of lipases from *Bulkhorderea cepaceae* and *Pseudomonas fluorescens*. Ultrasound accelerated the cross-linking reaction while preserving the biocatalytic activity of CD-hybrids. The promising biocatalytic activities of these newly designed immobilized enzymes have been illustrated in the esterification of free fatty acids for biodiesel production and monostearin synthesis.

Experimental Section

Materials

The free and immobilized lipases from *Bulkhorderea cepaceae* (Amano PS) and *Pseudomonas fluorescens* (AK Amano) were obtained from Amano. *n*-Heptane and oleic acid were purchased from Tedia Co., while (*R,S*)-1,2-isopropylidene glycerol and all chromatographic standards were purchased from Sigma-Aldrich. Stearic acid (>98%) and ethanol were purchased from Vetec Ltda. All other chemicals were purchased from Alfa-Aesar Italy and used without further purification. β-CD was kindly provided by Wacker Chemie (Germany). Ultrasound-assisted cross-linking reactions were carried out in an ultrasonic bath (35 kHz, Transsonic 460, Elma).

GC-MS Analysis

All GC-MS analyses were performed using the EN 14105 modified method. Free fatty acids and (R,S)-1,2-isopropylidene glycerol were transformed into more the volatile silylated derivatives in the presence of pyridine and *N*-methyl-*N*-trimethysilyl trifluoroacetamide (MSTFA). All GC-MS measurements were carried out in duplicate using a DB 5-HT (Agilent, J & W. Scientific, U.S.A.) capillary column (10 m x 0.32 mm x 0.1 μm). Quantification was conducted on the basis of calibration curves with internal standards. GC-MS samples were prepared by dissolving 0.1 g of the final product in 1 mL of n-heptane. 100 μL of this solution and pyridine solutions of butanetriol (1 mg/mL) and tricaprine (8 mg/mL), used as internal standards, were added to a flask that held 100

μL of MSTFA. After 15 min, these reactants were dissolved in 8 mL n-heptane. One micro-litre of this sample was then injected into Shimadzu CG2010 equipment.

Lowry-Tinsley Analysis

Esterification measurements were performed using a modification of the Lowry-Tinsley assay. Fatty acid depletion was monitored as follows; 0.30 mL of the reaction solution, including buffer solutions, was added to a tube containing 0.6 mL of *n*-heptane and 1 mL of cupric acetate-pyridine (5% w/v, pH 6.0). The final solutions were vigorously mixed for 30 s in a vortex shaker and the upper organic phase was measured on a UV/visible spectrophotometer at 715 nm. Each reaction was analyzed in triplicate and content conversion was calculated according to the absorbance percentage difference shown by the stock solution.

Enzyme Immobilization on HDI cross linked β-CD

β-CD reticulation was performed using 1,6-diisocyanatohexane (HDI) as an efficient cross-linker in the presence of PS-AMANO or AK-AMANO enzymes (10% w/w).

β-CD (500 mg) was dissolved in DMF (7 mL) in a 100 mL three-necked round bottom flask and the addition of the corresponding enzyme was preceded by the addition of HDI (800 μL). The mixture was magnetically stirred for 12 h at room temperature and then sonicated at 30 °C for 2 h in an ultrasonic bath (35 kHz, 30 W). The resulting reticulation product (a white solid gel – Figure 1) was transferred to a mortar, mildly grinded and washed twice with acetone (100 mL), MeOH (100 mL), and water (100 mL). The obtained product was lyophilized and stored as a white powder under a nitrogen atmosphere.

Esterification Activity Assay

Oleic acid and butanol were used as substrates for the esterification reaction. Every mL of *n*-heptane solution contained an equimolar mixture of substrates (0.1 mol^{-1}), as well as the free or immobilized lipase (10mg/mL). The reaction mixture (40°C) was stirred at 200 rpm in 1mL vials. 300μL aliquots were taken at intervals and residual fatty acid levels were analyzed using the previously described Lowry-Tinsley method.

Specific esterification activity (mmol · min^{-1} · g) was determined by calculating the conversion of fatty acid to ester and defined as micromoles per hour per milligram of protein, according to Cao *et al*.. [20]

3. Results and discussion

Highly cross-linked CDs are insoluble polymers obtained from the reaction of CDs with a series of bi-functional reagents.[21] These include diphenylcarbonate, epichlorohydrin, diisocyanates and the ultrasound-promoted copper-catalyzed azide-alkyne cycloaddition of a randomly propargylated β-CD with 1,3-bis(azidomethyl)benzene.[22] Our proposed approach deals with the utilization of solid cross-linked β-CDs as enzyme supports Cross-linked CDs could be obtained at room temperature under mild sonication via reticulation using hexamethylene diisocyanate (HDI).

A range of typical solvents were screened in the esterification experiments (Table 1). The esterification activities of free and commercial immobilized lipases from *Pseudomonas cepaceae* and *Bulkholderia cepaceae* with AKβCD and PSβCD were initially compared.

Table 1. Esterification activity of free and immobilized lipases.

Organic Solvents	**Esterification Activity (mmol · min^{-1}· mg)**					
	AK	AKIM	AKβCD	PS	PSIM	PSβCD
***n*-hexane**	120.22	139.34	191.56	145.71	204.44	223.45
***n*-heptane**	119.56	150.32	198.72	155.23	208.33	221.97
***iso*-octane**	99.43	125.34	155.44	111.19	165.46	201.75
MTBE	98.51	112.5	121.33	100.06	128.19	178.68
ciclohexane	110.65	137.29	165.87	134.12	173.08	203.65

[a]AK: *Pseudomonas fluorescens*; AKIM immobilized AK, AKbCD: AKimmobilized on crosslinked b-CD. [b] PS: *Bulkholderia cepaceae*; PSIM immobilized PS, PSβCD: PS immobilized on crosslinked β-CD. Reaction conditions: oleic acid and butanol 1 : 1 (0.1 mol^{-1}) in nheptane, 40 °C and 200 rpm, with 10 mg mL^{-1} of biocatalyst

Results clearly demonstrate that all types of immobilized lipases exhibited improved esterification activity over the free enzymes regardless of the utilized solvents. Interestingly, both AK and PSβCD showed superior activity in hydrocarbon solvents (e.g. *n*-hexane and *n*-heptane). AKβCD and PSβCD showed higher esterification activity as

compared to that of the corresponding commercial immobilized lipases, which demonstrates the remarkable advantages of the proposed immobilization protocol.

The thermal stability of immobilized lipases AKIM, PSIM, AKβCD and PSβCD was further investigated. The reactions were conducted under identical conditions to those selected for the esterification activity, although numerous temperatures, varying from 30 to 70°C, were used (Figure 1).

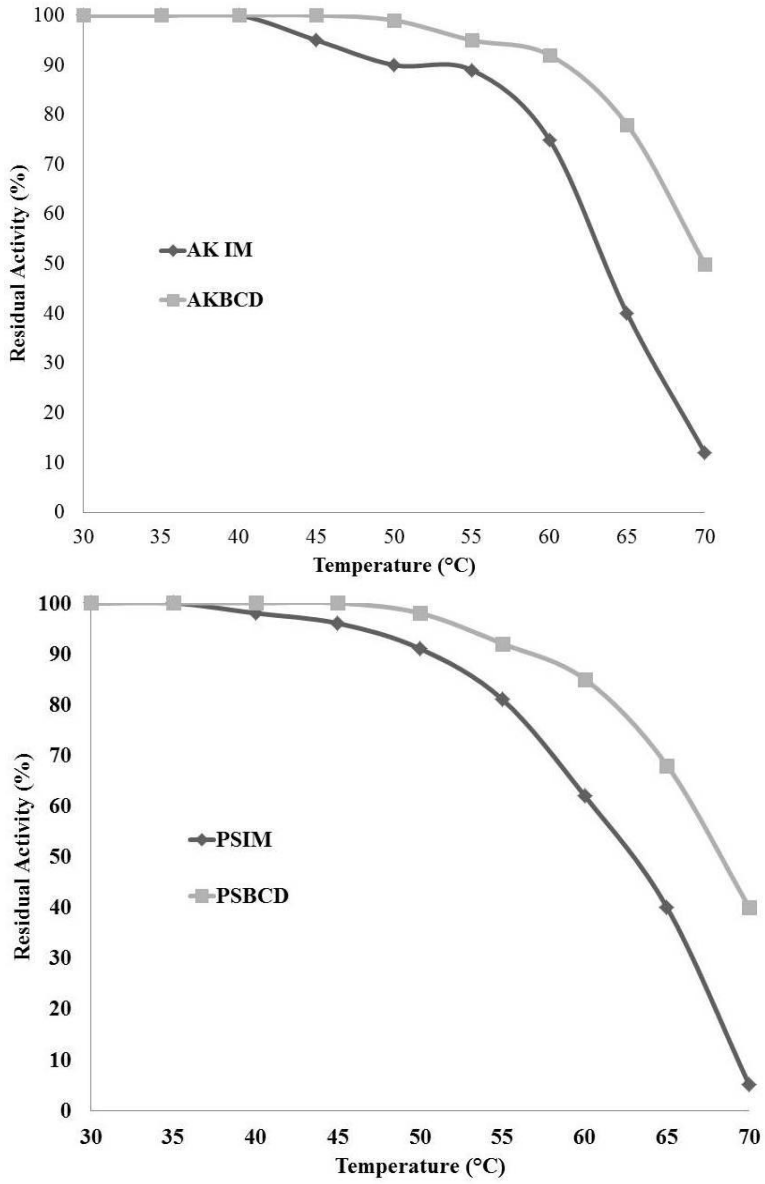


Figure 1. Thermal stability of immobilized lipases.

Results depicted in Figure 1 show the improved stability of immobilized AKβCD and PSβCD with respect to commercial immobilized enzymes. Firstly, the commercial

AKIM exhibited a significant loss in activity (e.g. over 25% total activity loss at 60°C). Comparably, AKβCD maintained almost quantitative esterification activity up to 60°C, with a clearly noticeable improvedthermo-resistance. Similar profiles were found for PSβCD lipase. The commercial immobilized PSIM starts losing activity sharply at 45°C, while PSβCD presented 100% activity at the same temperature. In this preparation, a substantial decrease in esterification activity was only observed above 55°C.

In order to obtain better insights into the observed improved activities and stabilities of β-CD immobilized enzymes, a series of characterization studies were conducted. These included XRD, TG-DTA and XPS. Results have been depicted in Figures 2 and 3 (see also ESI). XRD data point to β-CD material amorphisation upon cross-linking and enzyme immobilization (see also ESI). Important differences were also observed in TG-DTA experiments (Figure 2). These clearly indicate two distinctive steps of mass loss associated with β-CD decomposition (present in all systems at ca. 330ºC) with an additional significant mass loss in the 450-550ºC range, which seemed to be associated with enzyme removal. The high decomposition temperature may be indicative of a strong enzyme-β-CD interaction. [23]

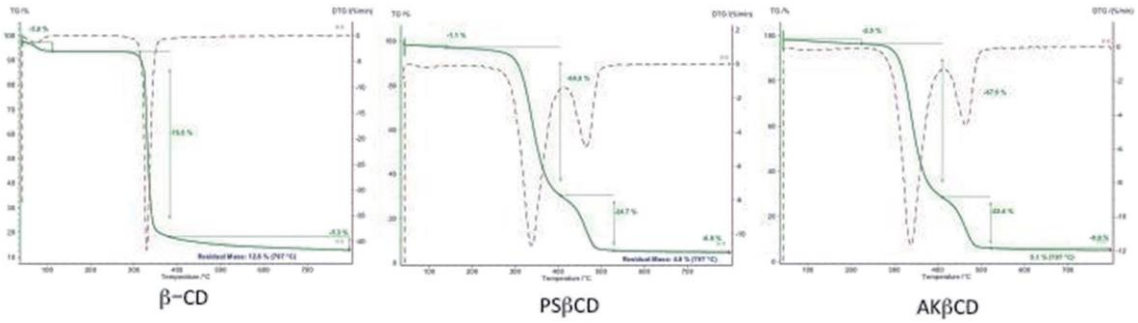


Figure 2. Please Include TG profiles of BCD and a PS or AK-Amano β-CD

This possible strong enzyme-β-CD interaction supports activity data obtained for β-CD biocatalytic hybrids (Table 1, Figure 1). XPS results were also in good agreement with TG-DTA data and confirmed the presence of the enzyme in β-CD cross-linked systems as indicated by the number and proportion of C species in the material (Figure 3). N could also be detected in AK and PSβCD materials (as compared to blank β-CD samples, see ESI). N is likely to come from the peptide bonds present in the protein, while this was obviously not detected in BCD.

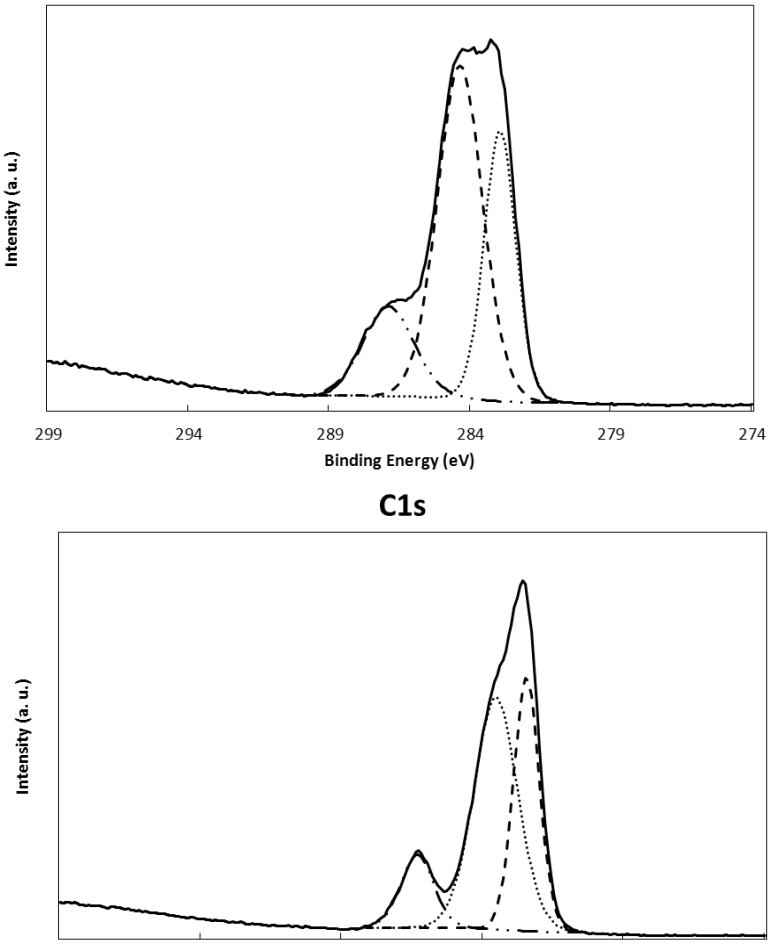


Figure 3. C1s spectra of AKBCD (top) and BCD (bottom) showing significantly different C contributions.

Biocatalytic applications

The observed enzymatic activity enhancement under the proposed immobilization methodology was subsequently extended to previously optimized processes.[24] Firstly, the esterification of oleic acid and ethanol was conducted according to previously optimised conditions by Costa *et al.* 2011 (Figure 4). [24]

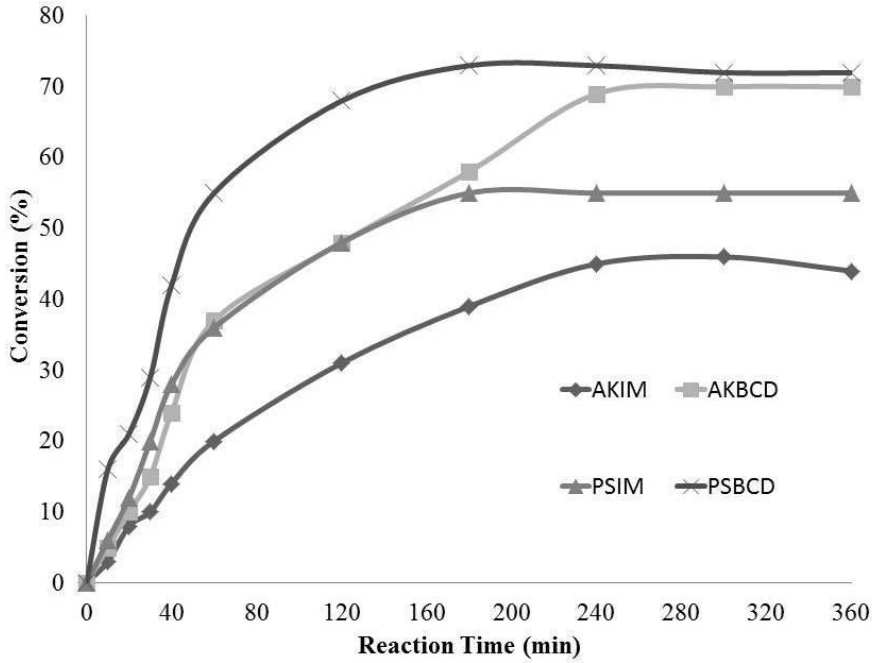
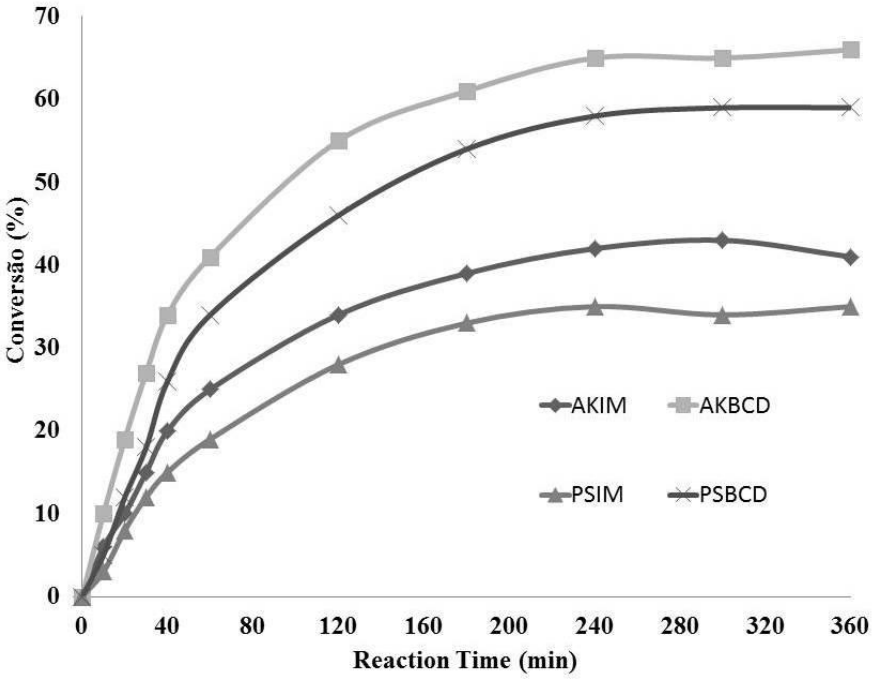


Figure 4. Ethyl oleate synthesis catalyzed by immobilized lipases.

Results from Figure 4 point to a significantly improved performance of immobilized lipases AKβCD and PSβCD with respect to the immobilized commercial PSIM and AKIM enzymes from Amano. The latter two exhibited regular conversions in 4h; 39% for AKIM and 51% for PSIM. Interestingly, reaction equilibrium was achieved after 3h, with a 72% conversion rate, when this reaction was catalyzed by PSβCD. Similar results were achieved only after 4h with AKβCD.

In another application, monostearin synthesis was attempted following previously optimized conditions by by Junior *et al*., 2012.[24]

Fig 5:



Monostearin synthesis catalyzed by immobilized lipases.

As a result, our new biocatalysts were able to induce good conversions in 4 hours, 62% by AKβCD and 53% by PSβCD, which are significant improvements over PSIM and AKIM lipases.

In conclusion, the development of highly active and stable novel immobilised enzymes using a simple and efficient crosslinking protocol with beta-cyclodextrins was successfully accomplished, rendering versatile biocatalysts for different applications including esterifications. We envisage this methodology to be extended to a number of related chemistries of relevance to waste valorisation to a range of valuable products that will be reported in due course. The reusability of the biocatalyst was also evaluated and expressed in terms of monoacylglycerol conversion as depicted in Fig. 6. As observed, both biocatalysts were able to maintain a high performance and stability during 9 cycles. Comparably, a significant decrease in conversion was observed for AKBCD with respect to PSBCD after 9 uses. Interestingly, commercial lipases showed both reduced activities and a gradual decrease in activity after 6–7 reuses.

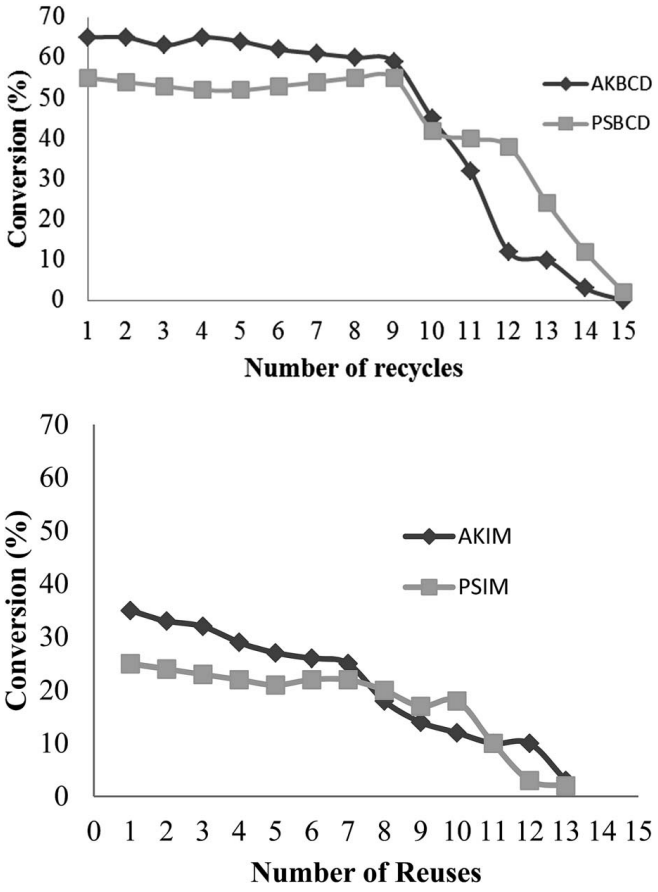


Fig.6 Recycles of new immobilized biocatalysts. (A) New immobilized lipases. (B) Commercial lipases

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